Comparison of Two Commercial Tick-Borne Encephalitis Virus IgG Enzyme-Linked Immunosorbent Assays

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Despite the availability of protective vaccines, tick-borne encephalitis virus (TBEV) infections have been increasingly reported to the European Centre for Disease Prevention and Control in the past 2 decades. Since the diagnosis of TBEV exposure relies on serological testing, we compared two commercial enzyme-linked immunosorbent assays (ELISAs), i.e., Immunozym FSME IgG assay (ELISA-1) and Euroimmun FSME Vienna IgG assay (ELISA-2). Both assays use whole TBEV antigens, but they differ in viral strains (Neudoerfl for ELISA-1 and K23 for ELISA-2) and cutoff values. In testing of samples from 398 healthy blood donors, ELISA-1 showed higher reactivity levels than ELISA-2 ($P < 0.001$), suggesting different assay properties. This finding was supported by Bland-Altman analysis of the optical density at 450 nm (OD_{450}) (mean bias, $+0.32$ [95% limits of agreement, -0.31 to $+0.95$]) and persisted after transformation into Vienna units. Concordant results were observed for 276 sera (69%) (44 positive and 232 negative results). Discordant results were observed for 122 sera (31%); 15 were fully discordant, all being ELISA-1 positive and ELISA-2 negative, and 107 were partially discordant (101 being ELISA-1 indeterminate and ELISA-2 negative and 6 having positive or indeterminate reactivity in both ELISAs). Neutralization testing at a 1:10 dilution yielded positive results for 33 of 44 concordant positive sera, 1 of 15 fully discordant sera, and 1 of 33 partially discordant sera. Indirect immunofluorescence testing revealed high antibody titers of ≥100 for yellow fever virus in 18 cases and for dengue virus in one case, suggesting that cross-reactivity contributed to the ELISA-1 results. We conclude that (i) cross-reactivity among flaviviruses remains a limitation of TBEV serological testing, (ii) ELISA-2 revealed reasonable sensitivity and specificity for anti-TBEV IgG population screening of human sera, and (iii) neutralization testing is most specific and should be reserved for selective questions.

Despite the availability of protective vaccines, tick-borne encephalitis (TBE) has been increasingly reported in Europe over the past 2 decades (1). According to a recent analysis by the European Centre for Disease Prevention and Control (ECDC), 29,381 cases of TBE have been diagnosed in 16 countries of the European Union (EU)/European Free Trade Association (EFTA) since the year 2000. The annual rates range from 2,000 to 3,500 cases per year, showing a net increase despite a remarkable and as yet unexplained 3-year peak periodicity. Climate changes, with milder winters and earlier and prolonged summer seasons, and increasing numbers of small rodent and larger wild and domestic animal hosts, such as deer, sheep, and goats, are thought to contribute to expanding areas in which tick-borne encephalitis virus (TBEV)-infected ticks are endemic, which facilitates human exposure during occupational and leisure activities outdoors (2–4). The notable exception to this European trend is Austria, where TBEV vaccination has been effectively delivered (5). In Switzerland, TBEV-infected ticks have been documented to expand to new geographic localities, with now close to 40 foci of endemicity and a mean prevalence of TBEV-infected ticks of 0.46% (6).

TBEV belongs to the family *Flaviviridae*, genus *Flavivirus*, and includes the European, Siberian, and Far Eastern subtypes, with their respective geographic distributions (7). TBEV is transmitted to humans through the saliva of infected ticks. *Ixodes ricinus* is the main vector of TBEV in central, northern, and eastern Europe and *Ixodes persulcatus* in parts of the Baltic States, Finland, Russia, and Siberia. Rarely, other routes of transmission, such as consumption of raw milk products from viremic livestock, have been implicated (8, 9). The clinical manifestations of TBEV infections are typically biphasic, with a nonspecific flu-like viral syndrome during the initial viremic phase followed by meningitis, encephalitis, and me-

ningoencephalitis in the second phase, during which viremia is cleared and TBEV-specific antibodies are mounted. Thus, TBE is usually diagnosed during the second phase by serological testing.

TBE recently has become a notifiable disease according to ECDC recommendations, but there are still wide variations in national case definitions and reporting practices. Moreover, serological testing is not standardized, although the quantification of TBEV antibody activity (e.g., in Vienna units [VIEU]) has been attempted. In order to complement current surveillance studies on TBEV prevalence rates in ticks, baseline seroprevalence studies in healthy individuals are of interest, but robust assays are necessary for this purpose. Here we compared two commercially available enzyme-linked immunosorbent assays (ELISAs) among 398 healthy blood donors in Basel, Switzerland, and we report on test performance and concordance of results.
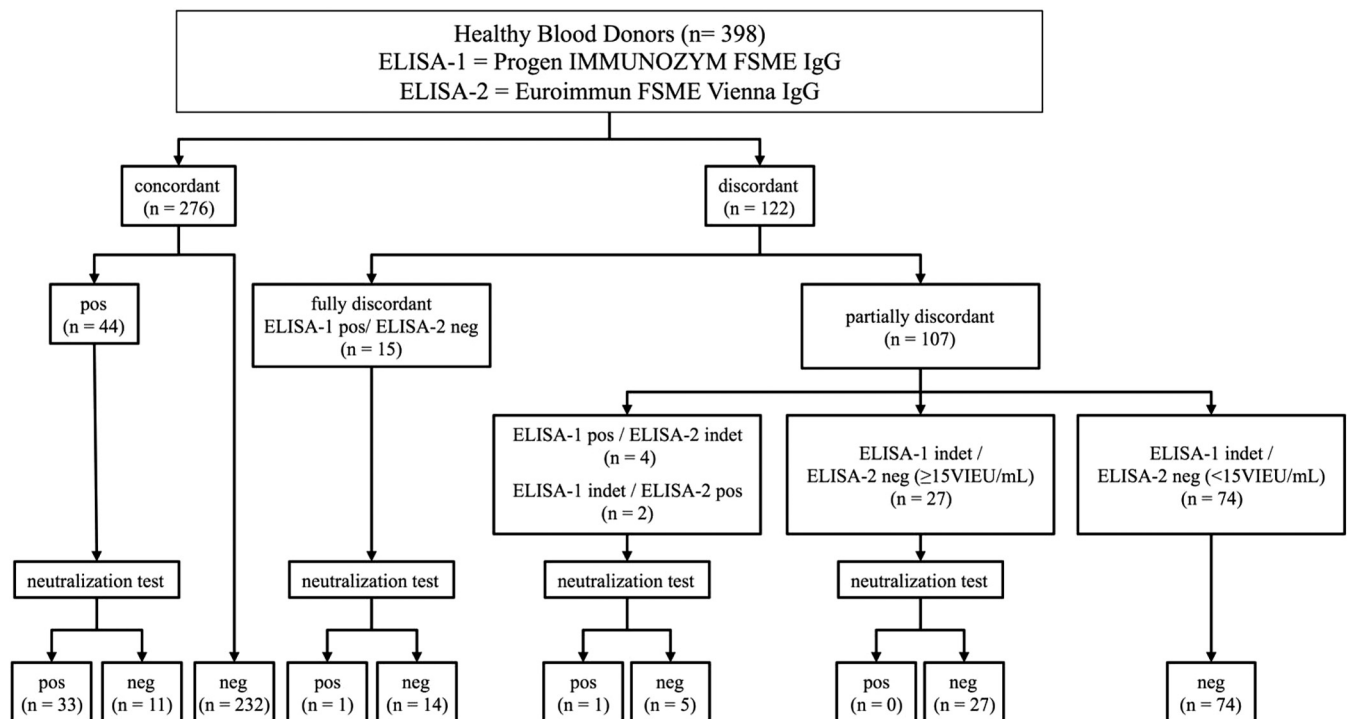
FIG 1 Testing flowchart. Serum samples from 398 healthy blood donors were tested for anti-TBEV IgG by the indicated ELISAs. Sera with concordant positive results or any discordant results of ≥15 Vienna units (VIEU)/ml in ELISA-2 were tested by neutralization testing (NT). pos, positive; neg, negative; indet, indeterminate.

MATERIALS AND METHODS

Blood donor population. Serum samples from 398 healthy blood donors were collected between February 2007 and January 2008, as described previously (10) (Fig. 1). During the past 5 years, samples underwent three cycles of freezing and thawing. The stability of the antibody activities was independently confirmed by ELISA in another study (11). All sera were stored at −20°C; aliquots of the samples were thawed and stored at 4°C during the testing period and were refrozen afterwards.

TBEV-specific IgG ELISAs. ELISA-1 was the Immunozym FSME IgG assay (lot A13008; Progen, Heidelberg, Germany), which is the current routine test in the Division of Infection Diagnostics, a fully accredited microbiology laboratory (according to ISO/IEC 17025:2005 criteria). ELISA-1 uses TBEV antigens prepared from Neudoerfl strain-infected cell cultures. The optical density at 450 nm (OD_{450}) was measured, and results were transformed into VIEU per milliliter using one-point calibration (calibrator 4), low-level and high-level positive serum samples, and a Microsoft Excel module (Immunozym one-point calibration module). The lower and upper limits of quantification were 15 VIEU/ml and 340 VIEU/ml, respectively, and OD_{450} values higher than the upper limit were restricted to the upper limit value, as described by the manufacturer. The results were categorized as negative for values of <63 VIEU/ml, indeterminate for values between 63 and 126 VIEU/ml, and positive for values of >126 VIEU/ml.

ELISA-2 was the FSME Vienna IgG assay (lot E130723AN; Euroimmun, Lübeck, Germany), which used TBEV antigens prepared from K23 strain-infected cell cultures. The OD_{450} values were transformed into VIEU per milliliter using a reference curve derived from calibrators 1 to 4, as described by the manufacturer. The lower and upper limits of quantification were 15 VIEU/ml and 1,000 VIEU/ml, respectively. According to the manufacturer, the linear range of ELISA-2 was between 81 and 978 VIEU/ml. Values exceeding the upper limit of quantification were not restricted, but samples could be retested using a higher predilution. The results were categorized as negative for values of <120 VIEU/ml, indeter-

minate for values between 120 VIEU/ml and 165 VIEU/ml, and positive for values of ≥165 VIEU/ml. ELISA-2 was performed with the DS2 ELISA automation system (Dynex Technologies, Chantilly, VA), according to the manufacturer's protocols and the distributor's advice (RUWAG Handels AG, Bettlach, Switzerland).

Neutralization testing. Selected sera, including 44 concordant ELISA-positive samples, 15 fully discordant samples (ELISA-1 positive/ELISA-2 negative or ELISA-1 negative/ELISA-2 positive), and 33 partially discordant samples (any other discordant combination of ELISA-1 and ELISA-2 results, i.e., indeterminate/positive, indeterminate/negative, positive/indeterminate, or negative/indeterminate) were sent to the Department of Virology, Medical University of Vienna (Vienna, Austria), and the presence of TBEV-neutralizing IgG antibodies was tested in 2-fold dilutions starting at 1:10, as described elsewhere (12).

Indirect immunofluorescence testing. Selected sera, including 15 samples with fully discordant results and 33 with partially discordant results for ELISA-1 and ELISA-2, were tested for other flavivirus reactivities by indirect immunofluorescence (indIF) testing (Euroimmun flavivirus mosaic) at Euroimmun AG (Lübeck, Germany). All sera were tested at dilutions of 1:10, 1:100, 1:1,000, 1:32, and 1:320, to derive the most likely specific response from the highest titer.

Statistical analysis. All results were collected in data groups and analyzed by GraphPad Prism software (version 6.0d). Non-Gaussian distribution was assumed for samples yielding P values of <0.05 in D'Agostino-Pearson (omnibus K2 test), Shapiro-Wilk, and Kolmogorov-Smirnov normality tests.

Paired groups were tested with the Wilcoxon matched-pairs signed-rank test or the chi-square test. Significant differences were assumed for P values of <0.05. Bland-Altman analysis and plots were used to compare paired results, and findings are expressed as mean bias differences with 95% limits of agreement (13). Contingency tables were analyzed with the κ coefficient using GraphPad QuickCalcs (http://graphpad.com/quickcalcs/kappa2) or with Cramer's V (φ_c) using the VassarStats website

TABLE 1 Intra-assay precision ($n = 6$)

Test and sample	Primary results[a]		Transformed results	
	OD$_{450}$ (mean ± SD)	CV (%)	VIEU/ml (mean ± SD)	CV (%)
ELISA-1				
High-level control	1.72 ± 0.153	8.89	>340	NA
Low-level control	0.73 ± 0.139	18.97	166.1 ± 20.7	12.45
Calibrator 4	1.99 ± 0.196	9.83		
ELISA-2				
Positive control	1.39 ± 0.085	6.13	760.0 ± 20.2	2.66
Negative control	0.09 ± 0.042	48.42	30.3 ± 18.0	59.46
Calibrator 1	1.74 ± 0.103	5.94		
Calibrator 2	0.74 ± 0.048	6.48		
Calibrator 3	0.41 ± 0.026	6.40		
Calibrator 4	0.05 ± 0.003	6.05		

[a] OD$_{450}$, optical density at 450 nm; SD, standard deviation; CV, coefficient of variation; NA, not applicable.

(http://vassarstats.net/newcs.html). Median results were presented with the 25% to 75% percentiles, to indicate the interquartile range (IQR).

RESULTS

ELISA reproducibility of calibrators. We investigated the intra-assay precision among controls and calibrators used for each run. The antibody activity, measured as OD$_{450}$, and the transformed VIEU/ml values were analyzed for six measurements (Table 1). For ELISA-1, the coefficients of variation (CVs) of the OD$_{450}$ values showed excellent precision of <10% except for the 18.97% for the low-level control samples. Similarly, the VIEU-transformed data for the low-level control samples yielded 12.45%, indicating sufficient precision within the transformed data. The high-level control value was above 340 VIEU/ml and was restricted, as indicated by the manufacturer. For ELISA-2, the four calibrators and the positive control showed excellent precision, with CVs of <10%. ELISA-2 also provided a negative control, which showed a CV of 48.42% (59.46% after transformation into VIEU), as expected for very low signals. The data indicated acceptable precision for both assays.

Comparison of TBEV antibody activities in ELISA-1 and ELISA-2. The sera of 398 healthy blood donors were tested for IgG with both ELISA-1 and ELISA-2, as recommended by the respective manufacturers. Comparison of the antibody activities (expressed as OD$_{450}$ values) revealed a significantly higher median OD$_{450}$ for ELISA-1 (OD$_{450}$, 0.26 [IQR, 0.18 to 0.40]) than for ELISA-2 (OD$_{450}$, 0.03 [IQR, 0.02 to 0.06]; $P < 0.0001$) (Fig. 2A). The data suggested significant differences in the assay properties. To compare the individual results, a Bland-Altman analysis was performed, whereby, for each serum sample, the (ELISA-1 result − ELISA-2 result) difference was plotted against the arithmetic mean of both assays (i.e., [ELISA-1 result + ELISA-2 result]/2) (Fig. 2B). As illustrated by the graph, ELISA-1 yielded steadily higher OD$_{450}$ values than did ELISA-2, with a mean bias difference in OD$_{450}$ values of +0.32 (95% limits of agreement, −0.31 to +0.95).

Since both assays provide quantification of antibody activity through the use of calibrators, we compared transformed data in VIEU per milliliter, as recommended by the respective manufacturers. The median IgG level for ELISA-1 was 57 VIEU/ml (IQR, 38 to 89 VIEU/ml), which was significantly higher than the median level for ELISA-2* of 15 VIEU/ml (IQR, 15 to 18 VIEU/ml; $P < 0.0001$) (Fig. 3A). Since anti-TBEV IgG quantification by ELISA-1 was restricted to a maximum value of 340 VIEU/ml, we also restricted ELISA-2 results to 340 VIEU/ml (Fig. 3A, ELISA-2*); however, the difference between the two activities remained significant ($P < 0.0001$). To compare the individual transformed VIEU results, a Bland-Altman analysis was performed, yielding a mean bias difference of +37 VIEU/ml (95% limits of agreement, −33 to +107 VIEU/ml). Thus, the results indicated that the ELISAs yielded significantly different quantifications of TBEV IgG activities, with ELISA-1 values being higher for almost all sera tested.
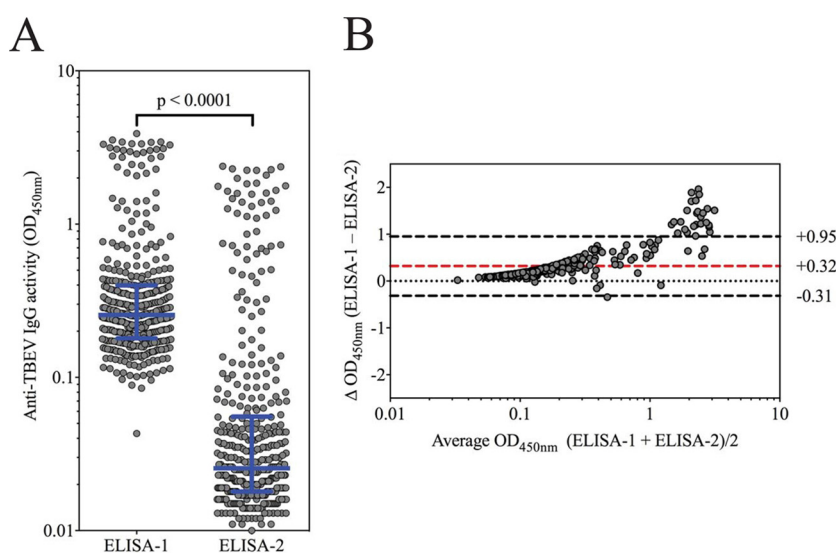

FIG 2 Comparison of anti-TBEV IgG activities determined by ELISA-1 and ELISA-2. (A) Primary readout (OD$_{450}$) data for sera from 398 healthy blood donors that were tested by ELISA-1 and ELISA-2. Lines, respective medians and 25th and 75th percentile values (Wilcoxon test, $P < 0.0001$). (B) Bland-Altman plot of the OD$_{450}$ results shown in panel A. Dashed black lines indicate the 95% limits of agreement; the dashed red line indicates mean bias difference.
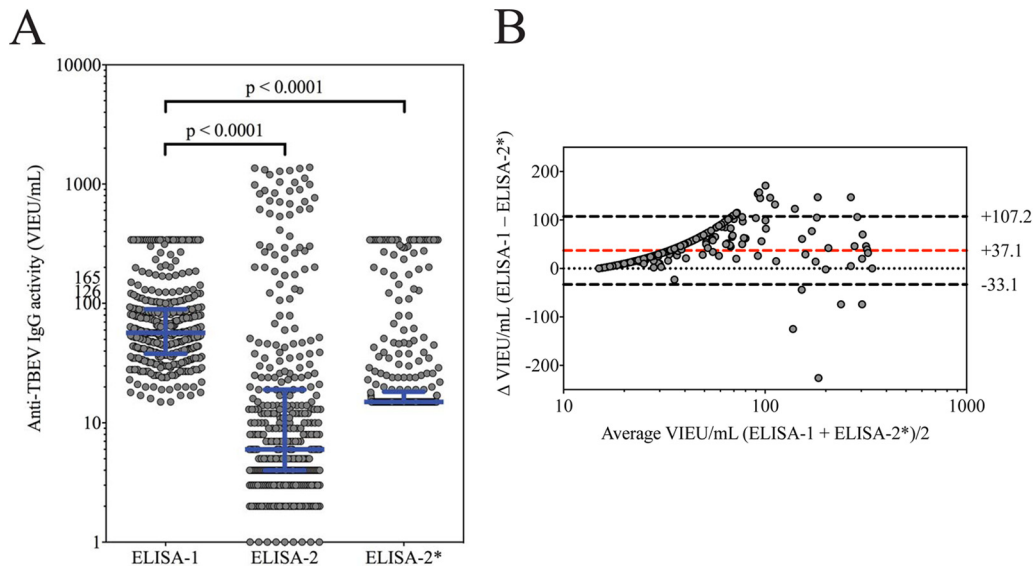
FIG 3 Comparison of anti-TBEV IgG values transformed into Vienna units according to ELISA-1 and ELISA-2 calibrators. (A) Transformed data for sera from 398 healthy blood donors that were tested by ELISA-1 and ELISA-2. The results were transformed into VIEU per milliliter as described by the manufacturers. The ELISA-1 results were limited to a ceiling of 340 VIEU/ml. ELISA-2 results are expressed without a corresponding ceiling; for ELISA-2* results, the value of 340 VIEU/ml used for ELISA-1 was applied. Lines, respective medians and 25th and 75th percentiles (Wilcoxon $P < 0.0001$). (B) Bland-Altman plot of the ELISA-1 and ELISA-2* VIEU/ml results shown in panel A. Dashed black lines indicate the 95% limits of agreement; the dashed red line indicates mean bias difference.

Comparison of qualitative results. To investigate the baseline seroprevalence among healthy blood donors, the quantitative results were categorized as recommended by the respective manufacturers. The frequency plot of the numbers of samples within 15-VIEU/ml intervals indicated that 294 samples (74%) were below the lower limit of quantification of 15 VIEU/ml for ELISA-2 (Fig. 4). Another 49 samples (12%) had TBEV IgG levels below 63 VIEU/ml and 9 (2%) had activities between 63 and 165 VIEU/ml (i.e., negative and indeterminate, respectively) by ELISA-2. In contrast, ELISA-1 had only 2 samples (1%) with activities below 15 VIEU/ml; a large group of 230 samples (58%) had levels below 63 VIEU/ml and hence were called negative, and another 103 samples (26%) had activities in the indeterminate range between 63 and 126 VIEU/ml (Fig. 4). Thus, ELISA-1 and ELISA-2 differed

not only in TBEV IgG quantification values but also in the qualitative categories of negative, indeterminate, and positive sera.

To investigate concordance between the two assays, the definitions for the respective ELISAs were applied (Table 2). Concordant results were obtained for 276 sera (69.3%), including 44 positive (11.0%) and 232 negative (58.3%) sera. Discordant results were obtained for 122 sera (30.7%). These results included 15 fully discordant results, all being ELISA-1 positive (i.e., >126 VIEU/ml; median, 167 VIEU/ml [IQR, 130 to 179 VIEU/ml]) but ELISA-2 negative (i.e., <120 VIEU/ml; median, 29 VIEU/ml [IQR, 10 to 51 VIEU/ml]), and 107 partially discordant results, including 6 sera with intermediate or positive reactivity in both assays and 101 sera with indeterminate ELISA-1 results (median, 84 VIEU/ml [IQR, 73.5 to 102.0 VIEU/ml]) but negative ELISA-2
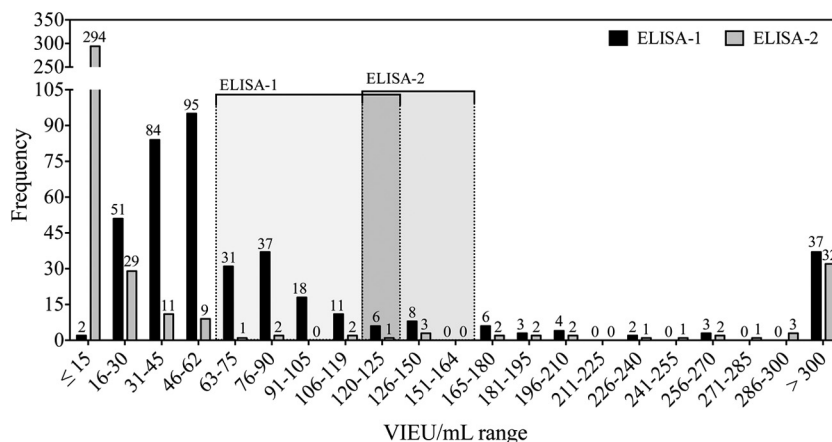

FIG 4 Frequency histograms of TBEV IgG Vienna unit distribution. The numbers of sera falling into the indicated categories were plotted for ELISA-1 and ELISA-2. Gray zones, indeterminate results for ELISA-1 (left) and ELISA-2 (right).

TABLE 2 Comparison of ELISA-1 and ELISA-2 results

| ELISA-2 result | No. of samples | | | |
	ELISA-1 positive	ELISA-1 indeterminate	ELISA-1 negative	Total
Positive	44	2	0	46
Indeterminate	4	0	0	4
Negative	15	101	232	348
Total	63	103	232	398

results (median, 7 VIEU/ml [IQR, 4.0 to 16.5 VIEU/ml]). Of the latter, 74 sera had levels of <15 VIEU/ml by ELISA-2 and were accepted as TBEV IgG negative. Repeat testing was performed for the 15 fully discordant sera, the 27 sera that were ELISA-1 indeterminate but ELISA-2 negative (but ≥15 VIEU/ml), and the 6 sera with intermediate or positive reactivity in both assays (see Table S1 in the supplemental material). All retested sera remained discordant, but 7 and 4 sera changed ELISA-1 and ELISA-2 subgroups, respectively, from fully to partially discordant, as they had activities close to the respective VIEU/ml limits. Analysis in a 3 × 3 contingency table indicated that there were significant differences in the qualitative categories ($P < 0.0001$, chi-square test) and the agreement was only fair ($\kappa = 0.347$ [95% confidence interval [CI], 0.274 to 0.420]).

Neutralization testing. Selected sera of interest were submitted for neutralization testing (NT) at an external reference laboratory. Of the 44 concordant positive sera, 33 sera tested positive for TBEV neutralization at a dilution of ≥1:10. Among the 15 fully discordant sera, only 1 serum tested positive by NT. Among the 6 partially discordant sera with more than intermediate or positive results by ELISA-2, 1 tested positive by NT. Of the remaining 27 sera with more than 15 VIEU/ml by ELISA-2, no serum was NT positive (Fig. 1). The agreement between NT results and the categorical readouts was moderate for ELISA-1 ($\varphi_c = 0.545$) but substantial for ELISA-2 ($\varphi_c = 0.700$).

Characterization of discordant sera. To address the potential role of cross-reacting antibodies, we investigated 15 fully discordant and 33 partially discordant sera by indirect immunofluorescence, using a flavivirus panel containing yellow fever virus (YFV), West Nile virus (WNV), dengue viruses (DENVs) 1 through 4, and Japanese encephalitis virus (JEV). The highest titers of ≥100 were identified against YFV in 18 sera (38%). The highest titers against DENV were found in 2 sera (4%) and against WNV in 1 (2%). ELISA-1 results were mostly indeterminate or positive for these sera, whereas ELISA-2 results were mostly negative (see Table S1 in the supplemental material). These results suggest that cross-reactivity contributed more to the indeterminate results measured by ELISA and that ELISA-2 was more specific than ELISA-1.

Seroprevalence among healthy blood donors. The seroprevalence among healthy blood donors in Basel amounts to 15.3% (63/398 samples) according to ELISA-1 but only 11.6% (44/398 samples) according to ELISA-2. Using neutralizing antibodies detectable at a dilution of 1:10 as a reference, a seroprevalence of 8.8% (35/398 samples) must be assumed.

DISCUSSION

Serological testing for TBEV-specific antibodies is a key tool for TBE diagnosis, assessment of vaccine responses, and seroepide-

miological studies. The latter is gaining interest in view of the notion that, similar to other flavivirus infections, TBEV infections may not always lead to neurological disease and corresponding diagnostic evaluations. Given the increasing reports of symptomatic TBEV infections and the geographical expansion of TBEV-infected tick populations in Europe, complementing seroprevalence studies are of interest to better estimate TBEV exposure across different geographic areas and the changes over time.

The present study indicates that the two commercially available ELISAs differed significantly in their results on both quantitative and qualitative levels. On a quantitative level, both assays measure OD at 450 nm and provide transformations of anti-TBEV IgG activity in VIEU per milliliter, but both the primary and transformed results were significantly different. ELISA-1 results were significantly higher than ELISA-2 results for the entire population of healthy blood donors and for paired results in a Bland-Altman analysis, indicating a mean bias of +0.32 for OD_{450} values or +37.1 VIEU/ml. Although the manufacturer's protocol attempts to account for this higher reactivity of the ELISA-1 with a large indeterminate zone, only 69% concordant results with ELISA-2 were obtained. Conversely, 31% of the results were discordant, most of which (26% [$n = 103$]) showed indeterminate reactivity with ELISA-1 and negative reactivity with ELISA-2.

The reason for the discrepancy between ELISA-1 and ELISA-2 results is not entirely clear. The different TBEV strains used as antigens, i.e., Neudoerfl and K23, are unlikely to explain these differences, since both strains are highly conserved in their capsid proteins, and both are used as immunogens in vaccines. In fact, the degree of amino acid variation within European subtypes has been shown to be low, with a maximum divergence of 2.2% (7). The K23 and Neudoerfl strains are used in the European vaccines Encepur (Novartis Vaccines and Diagnostics GmbH & Co., Marburg, Germany) and FSME-Immun (Baxter AG, Vienna, Austria), respectively. Both vaccines induce neutralizing antibodies against all three subtypes of TBEV, but there might be some differences in reactivity depending on whether homologous or heterologous coating antigens were used for the vaccine (14, 15).

The transformation to VIEU could be a possible contributing factor, since these units are arbitrary (16) and not based on internationally validated and transferrable standard materials (such as, for example, the WHO-approved hepatitis B surface antibody standard units). However, the fact that the primary results expressed as OD_{450} values also showed these differences reduces the potential role of VIEU transformation.

More likely, the coated antigen preparation is less pure and might contain cross-reactive antigens other than mature capsid proteins in virions, which could come from the cell culture and include conserved nonstructural flaviviral proteins. This was also suggested by the frequency histogram (Fig. 4), which revealed a significant population of low-level reactive sera in the negative zone, tailing off into the indeterminate and positive zones, for ELISA-1. In contrast, the vast majority of sera classified as negative by ELISA-2 had activities of ≤15 VIEU/ml. The susceptibility to cross-reactivity of ELISA-1 is supported by the indirect immunofluorescence results for other flaviviruses. High titers for YFV, DENV, and WNV were associated with higher VIEU/ml values for ELISA-1 but not ELISA-2. Overall, ELISA-2 results showed greater agreement with NT results than did ELISA-1 results ($n = 92$; $\varphi_c = 0.700$ versus $\varphi_c = 0.545$). Our data suggest that ELISA-1 is more sensitive and ELISA-2 is more specific, with the reduced

specificity of ELISA-1 resulting in part from cross-reacting antibodies directed toward other flaviviruses. A recent external quality assessment (EQA) reported generally sufficient sensitivities for 15 commercial ELISAs and, in agreement with our findings, indicated that cross-reacting antibodies might impede reliable diagnostic statements. Furthermore, the need for an international standard was raised due to overall differences in cutoff values (17). The ELISAs evaluated in our study were included in the EQA, but sera with indeterminate results and larger sets of cross-reactive sera were not investigated.

Neutralizing antibodies are considered to be the best functional correlate of protection against TBEV infections. In our study, 33 of 44 sera with concordant positive results were NT positive at a dilution of 1:10, indicating that 75% of the positive test results were specific, with functional activity. It is unclear whether the 11 NT-negative sera must be considered falsely positive for the presence of TBEV-specific IgG or whether the sensitivity of this functional test at a dilution of 1:10 simply missed antibodies present at lower levels. In its present form, NT is costly and requires a specialized laboratory, both of which impede its use for larger-scale seroprevalence studies. Translating our results into TBEV seroprevalence rates among healthy blood donors in Basel, ELISA-1 yielded 15.8% positive results ($n = 63$), which increased to 41.7% when indeterminate results ($n = 166$) were included. ELISA-2 yielded 11.6% positive results ($n = 46$), which increased to only 12.6% when indeterminate results ($n = 50$) were included. NT identified a minimal seroprevalence estimate of 8.8%, close to the 11.6% value for ELISA-2.

Our study has some limitations that should be acknowledged. First, because of costs, not all sera were tested by neutralization testing, but we included those with at least one positive result or an indeterminate result and more than 15 VIEU/ml in either of the ELISAs. As discussed above, however, only 2 sera were identified in the latter population (4% [$n = 48$]). Taking into account the limited sensitivity of NT, the seroprevalence in this population is likely to be between the minimal NT estimate of only 35 NT-positive samples among the 398 samples (8.8%) and the ELISA-2 estimate of 11.6%. These rates correspond to earlier seroprevalence data from a geographically close area in the upper Rhine valley (18). A second drawback of our study is the lack of information on the vaccination or exposure histories of our population of healthy blood donors. The YFV responses in 18 individuals are most likely the results of vaccination, but the reactivity to DENV in 2 individuals and to WNV in 1 individual must be due to exposure, as vaccines were not available at the time of blood sampling. Finally, we note that the ELISAs were not used to evaluate patients with suspected TBEV-related disease. For such patients, higher sensitivity at the expense of specificity might be warranted, in order to avoid missed diagnoses. However, the search for less specific IgM in the acute second phase of TBE is not without problems (19), due to partly overlapping clinical presentations of other flavivirus infections, the increased potential of cross-reactivity, and the increasing risk of exposure to other flaviviruses through travel and now autochthonous transmission within Europe (20–22). This raises questions about confirmatory testing strategies.

We conclude that cross-reactivity among flaviviruses remains a limitation of TBEV serological testing that clinical virology laboratories and clinicians should consider. The ELISA-2 revealed reasonable sensitivity and specificity for population screening of human sera for anti-TBEV IgG. Neutralization testing remains the most specific assay and should be reserved for selective reference applications, including confirmatory testing and assessments of vaccine protection and failure (5, 23).

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